Transcriptional signature induced by a metastasis-promoting c-Src mutant in a human breast cell line

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Abstract: Deletions at the C-terminus of the proto-oncogene protein c-Src kinase are found in the viral oncogene protein v-Src as well as in some advanced human colon cancers. They are associated with increased kinase activity and cellular invasiveness. Here, we analyzed the mRNA expression signature of a constitutively active C-terminal mutant of c-Src, c-Src(mt), in comparison with its wild-type protein, c-Src(wt), in the human non-transformed breast epithelial cell line MCF-10A. We demonstrated previously that the mutant altered migratory and metastatic properties. Genome-wide transcriptome analysis revealed that c-Src(mt) de-regulated the expression levels of approximately 430 mRNAs whose gene products are mainly involved in the cellular processes of migration and adhesion, apoptosis and protein synthesis. 82.9% of these genes have previously been linked to cellular migration, while the others play roles in RNA transport and splicing processes, for instance. Consistent with the transcriptome data, cells expressing c-Src(mt), but not those expressing c-Src(wt), showed the capacity to metastasize into the lungs of mice in vivo. The mRNA expression profile of c-Src(mt)-expressing cells shows significant overlap with that of various primary human tumor samples, possibly reflecting elevated Src activity in some cancerous cells. Expression of c-Src(mt) led to elevated migratory potential. We used this model system to analyze the transcriptional changes associated with an invasive cellular phenotype. These genes and pathways de-regulated by c-Src(mt) may provide suitable biomarkers or targets of therapeutic approaches for metastatic cells. DATABASE: This project was submitted to the National Center for Biotechnology Information BioProject under ID PRJNA288540. The Illumina RNA-Seq reads are available in the National Center for Biotechnology Information Sequence Read Archive under study ID SRP060008 with accession numbers SRS977414 for MCF-10A cells, SRS977717 for mock cells, SRS978053 for c-Src(wt) cells and SRS978046 for c-Src(mt) cells.

DOI: https://doi.org/10.1111/febs.13694

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Transcriptional Signature Induced by a C-Terminal c-Src Mutant

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Abstract

Background: Deletions in the C-terminus of the proto-oncogene c-Src kinase that are found in the viral oncogene v-Src and some advanced human colon cancers have been associated with increased kinase activity and elevated cellular invasion. Here, we used the human breast epithelial cell line MCF-10A to analyze the gene expression signature of a constitutively active C-terminal mutant of c-Src, c-Src(mt), in comparison to the wildtype protein, c-Src(wt), which we characterized previously for its migration properties.

Results: Genome-wide transcriptome analysis revealed that c-Src(mt) deregulated the expression levels of about 430 genes that are mainly involved in the cellular processes of migration and adhesion, apoptosis and protein synthesis. More than 80% of these genes have previously been linked to cellular migration, while the others play roles, for instance, in RNA transport and splicing processes. Consistent with the transcriptome data, c-Src(mt)-, but not c-Src(wt)-expressing cells were able to migrate from blood vessels into lung tissue in a mouse model of tissue invasion *in vivo*. The gene expression profile of c-Src(mt)-expressing cells shows significant overlap with that of different primary human tumor samples, perhaps reflecting elevated Src activity in some cancerous cells.

Conclusions: Expression of c-Src(mt) lead to elevated migratory potential. We used this model system to analyze the transcriptional changes associated with an invasive cellular phenotype. The herein identified genes and pathways deregulated by c-Src(mt) might be exploited as biomarkers or therapeutic targets for metastatic cells.

Keywords: Src, Proto-oncogene, Migration, Invasion, Transcriptome

Background

The c-Src proto-oncogene is the cellular homolog of the viral oncogene v-Src of the Rous Sarcoma Virus (RSV). c-Src is a non-receptor tyrosine kinase involved in a wide range of cellular functions including migration, invasion and adhesion. Elevated Src activity promotes oncogenic transformation [1]. The *in vitro* transforming capacity of activated Src kinase has originally been shown in RSV-infected and thereby v-Src-expressing chicken cells [2]. Src kinase-induced transformation can also occur in mammalian cells. This has been shown, for instance, by ectopic expression of v-Src in mouse fibroblasts [3]. *In vitro* studies with the human non-transformed epithelial breast cell line MCF-10A [4] have shown that the activation of Src kinase activity leads to transformation *in vitro* and tumor formation in nude mice [5-7]. Despite its known role in promoting cellular transformation, many of the functions of Src on the molecular level remain unclear [1].

Known targets of Src kinase include focal adhesion proteins, adaptor proteins, cell cycle regulators and transcription factors. Src family kinases (SFKs) have been shown to modulate cell-cell and cell-matrix interactions and to promote the expression of matrix-degrading enzymes. The c-Src protein is regulated by reversible phosphorylation of tyrosine residues Y_{416} and Y_{527} (numbering according to v-Src) and by protein-protein interactions through its Src homology 2 (SH2) and SH3 domains [1]. Phosphorylation of Y_{527} inactivates the kinase activity of c-Src by inducing a compact repressed protein conformation, while its dephosphorylation leads to an open, active conformation [8,9]. Y_{527} is phosphorylated by non-receptor tyrosine kinases, the C-terminal Src kinase (CSK) and the CSK-homologous kinase (CHK) [10]. Dephosphorylation

of Y_{527} is catalyzed by several protein tyrosine phosphatases (PTPs) such as PTPα [11], SH2 domain-containing phosphatase-1 (SHP-1) [12] and SHP-2 [13]. Y_{416} is an autophosphorylation site whose phosphorylation is required for optimal c-Src kinase activity [14]. Moreover, various c-Src-interacting proteins can modulate its kinase activity non-enzymatically, including, for instance, focal adhesion kinase (FAK) [15] and SHP-2 [16] that bind to the SH2 and SH3 domains, respectively, leading to increased kinase activity.

A conserved C-terminal hydrophobic motif, GENL, is shared by a number of ubiquitously expressed SFKs, including Src, Yes and Fyn. In contrast, SFKs lacking this motif, Lyn, Lck, Hck and Blk, are mainly expressed in non-adherent hematopoietic cells [17]. We noticed that the C-terminus of c-Src was relevant for adherence and that its absence, for instance in the v-Src protein, seemed to contribute to non-adherence of cells. Therefore we suspected a possible role of the absent C-terminus in migration and invasion. The C-terminal GENL motif of c-Src has been described as binding motif for PDZ (postsynaptic density-95/discs large/zona occludens-1) domains, whereby the very C-terminal hydrophobic Leucine is crucial for this interaction. By substitution of this single amino acid from Leucine to Alanine, herein referred to c-Src(mt), we have recently demonstrated the loss of binding to PDZ domain-containing tumor suppressor proteins in MCF-10A and other cell lines. The c-Src-interacting PDZ proteins were the AF-6 (ALL-1 fused gene from chromosome 6), the membrane protein, palmitoylated 2, MPP2, a protein related to the *Drosophila* tumor suppressor discs large-A, dlg-A, and LNX1 (Ligand-of-Numb protein X1) [17-20]. Disruption of these interactions *via* alteration of the C-terminal motif leads to constitutive activation of the c-Src protein and elevated kinase activity compared to the wildtype c-Src protein with the intact GENL sequence [17-20]. We have previously noticed that

the expression of c-Src(mt) and, to a lesser extent, of c-Src(wt) changes the cellular morphology. This includes impaired cell polarization and disorganization of the cytoskeleton in MCF-10A cells associated with increased cellular transformation, which has been characterized by several *in vitro* assays [17,19]. The ectopic expression of c-Src(mt) in MCF-10A cells leads to increased colony formation in soft agar and foci formation on normal cell culture plates as well as elevated invasion of matrigel [17]. Moreover, c-Src(mt)-expressing cells show disruption of spheroid cell growth into acinar cultures in extracellular matrix, impaired wound healing and elevated migration on basement membranes *in vitro* [19].

To investigate the underlying gene expression changes, we here subjected the identical cellular model we used previously, MCF-10A cells expressing either c-Src(mt) or the wildtype c-Src(wt) [17-20], to genome-wide transcription profiling. This cellular model allows for delineating the effects of the single point mutation within the c-Src protein by comparison of otherwise genetically identically cell lines. We show that c-Src(mt), but not c-Src(wt), significantly deregulated the expression levels of 434 genes, herein referred as c-Src(mt) effector genes. These genes were characterized by means of pathways and biological functions, allowing detailed insights into the gene expression program of c-Src(mt). The gene products are mainly involved in migration and adhesion, apoptosis and protein synthesis. Increased migratory potential of c-Src(mt)-expressing cells was suggested by the gene expression profile and corroborated in an *in vivo* mouse model of lung tissue invasion, in line with our previous *in vitro* findings [17-20]. Literature mining revealed that about 20% of the c-Src(mt) effector genes have previously not been associated with cellular migration. These genes are mainly involved in RNA transport and splicing processes. Comparison of the gene expression profile of c-Src(mt)-

expressing cells with that of primary human cancer samples revealed significant overlap with about 60% of the tumor samples, indicating similarities between c-Src(mt)-expressing cells and some cancer cells that may have increased Src activity.

Results

Establishment of a c-Src(mt) cellular model

To study the effects of c-Src(mt), we selected the human non-transformed MCF-10A cell line stably expressing c-Src(mt), or c-Src(wt) as control [17-19]. The c-Src(mt) protein is identical to c-Src(wt) except for a single L-to-A substitution within the C-terminal GENL sequence. This motif mediates binding to PDZ domain-containing tumor suppressor proteins and is shared by the ubiquitously expressed SFKs Src, Yes and Fyn, but not by Fgr, Hck, Lck, Lyn and Blk expressed in non-adherent hematopoietic cells (Figure 1A). The c-Src protein contains Src homology 1 (SH1) or kinase, SH2 and SH3 domains (Figure 1B). The experimental approach comprised the generation of stable cell lines, RNA isolation, Illumina RNA sequencing (RNA-Seq), mapping of generated reads to the human genome with Bowtie [21], the quantification of expression levels on the transcript level through RNA-Seq by Expectation Maximization (RSEM) [22], determination of significantly differentially expressed (DE) genes with DEseq [23] and their functional characterization.

We stably transfected MCF-10A cells with either construct, c-Src(wt) or c-Src(mt), using lentiviral expression vectors (Additional file 1: Figure S1). Cells transfected with an otherwise identical expression vector but with a randomized nonsense sequence instead of c-Src were designated

mock cells. A tetracycline-inducible system allowed regulation of transgene expression by adding tetracycline (Tet) to the cell culture medium. Expression of the transgene in c-Src(wt)- and c-Src(mt)-transfected cells was verified by quantitative Reverse Transcriptase-PCR (qRT-PCR) (primer sequences can be found in Additional file 1: Table S1) and Western Blot (Figure 1C). RNA preparations displayed characteristic rRNA bands and no signs of degradation (Additional file 1: Figure S2). We selected those RNA preparations obtained from cells exposed to 1 µg/mL Tet for whole-genome expression profiling, as transgene expression was at physiological levels (Figure 1C). Of note, only c-Src(mt)-expressing cells exhibited a transformed phenotype upon exposure to Tet, including a stretched morphology, but no increased growth (data not shown). This is in accordance with our previous findings that expression of c-Src(mt) alters cellular morphology, but does not affect proliferation [19]. RNA preparations of all four cell lines were subjected to RNA-Seq, resulting in 99,100,544, 46,988,060, 38,071,856, and 56,106,630 (naïve MCF-10A, mock, c-Src(wt) and c-Src(mt), respectively) quality control-passed reads. Reads were mapped to the human genome with Bowtie [21], which yielded 76,735,649 (77.4%), 37,101,761 (79.0%), 30,474,883 (80.1%) and 43,473,640 (77.5%) mapped reads. Gene expression levels were then quantified on the transcript level with RSEM [22].

To learn about the global changes in gene expression induced through action of c-Src(wt) and c-Src(mt), expression levels of both cell lines were compared to those of naïve MCF-10A cells, using DEseq [23]. The comparison mock vs. naïve MCF-10A served as negative control. Resulting logarithmic fold change values (logFC) of significantly DE genes ($q \leq 0.05$) were plotted as density graphs (Figure 1D). While the profiles of mock and c-Src(wt) cells were almost identical and might reflect unspecific effects of the lentiviral transfection procedure, the profile of c-

Src(mt)-expressing cells was different and showed more pronounced gene expression changes in both directions (down- and upregulated genes), but comparable q values (Additional file 1: Figure S3) and a higher proportion of upregulated genes (Figure 1D). The similar density graphs of mock- and c-Src(wt)-expressing cells gave a first indication that c-Src(wt) was insufficient to induce detectable Src-specific gene expression changes, as noted before [17-20,24].

To verify Src activity, we performed qRT-PCR expression analysis of six known Src targets; p53 (*TP53*) [25] and the related p63 (*TP63*), Cyclin D2 (*CCND2*) [26] and mir-205 (*MIR205HG*) [27], all of which suppressed by Src, as well as matrix metalloproteinase-2 (*MMP2*) [28] and hyaluronan synthase 2 (*HAS2*) [29] (Additional file 1: Figure S4). *GAPDH* served as control, which as expected showed identical expression levels in all four cell lines. While the reported effects of Src could be verified for all of these known Src target genes in the c-Src(mt)-expressing cells, cycle threshold (Ct) values in the c-Src(wt)-expressing cells were similar to those of the naïve MCF-10A and mock cells. This gave further credence to the notion that expression of c-Src(wt) alone was insufficient to induce deregulation of Src targets in our cellular model – in contrast, expression of c-Src(mt) did suffice to alter the expression levels of these genes. This is in accordance with previous findings that either activating mutations [17-20,24] or co-factors [1] are required for increased Src activity. This might well be because c-Src(wt) is maintained in an inactivated state through binding of PDZ domain-containing tumor suppressor proteins to its C-terminal GENL sequence (Figure 1A). In contrast, c-Src(mt), due to mutation of this motif, has been shown to be impaired in binding to PDZ domains and is therefore constitutively active [19].

We next sought to investigate effector genes regulated by c-Src(mt). To account for possible Src-independent gene expression changes by the lentiviral transfection we selected the comparison

to mock cells instead of naïve MCF-10A cells as a more stringent control. This comparison, c-Src(mt) vs. mock, yielded a total of 434 significantly ($q \leq 0.05$) differentially expressed (DE) genes (Additional file 1: Table S2), herein referred to as c-Src(mt) effector genes, of which 52% (224 genes) were upregulated in the c-Src(mt)-expressing cells.

All known c-Src(mt) target genes analyzed above by qRT-PCR also showed significant differential expression levels between c-Src(mt) and mock cells inferred by RNA-Seq except for *TP53*, likely because the corresponding expression levels were too similar (Additional file 1: Table S2 and Figure S4). Three genes mentioned above, *TP63*, *CCND2* and *MIR205HG,* exhibited negative logarithmic fold change (logFC) values, whereas *MMP2* and *HAS2* showed positive logFC values with statistical significance, confirming our qRT-PCR results as well as previous findings [26-29]. Expectedly, *GAPDH* showed no altered expression. Interestingly, the expression level of endogenous c-Src was not altered, which was also seen in the Western Blot (Figure 1C). Therefore any effects on gene expression levels were due to activity of ectopically expressed c-Src(mt) and not of endogenous c-Src, which was expressed at identical levels in all four cell lines.

To further verify the RNA-Seq data, we selected eight additional genes with significant deregulation in the comparison c-Src(mt) vs. mock for qRT-PCR analysis (Additional file 1: Figure S5). logFC values were determined with the $2^{-\Delta\Delta CT}$ method and plotted against the logFC values obtained from RNA-Seq (Figure 1E). The strong correlation of R^2=0.983 verified the expression levels inferred by RNA-Seq.

In summary, the c-Src(mt)-expressing cells showed increased Src activity with previously reported effects on gene expression, and RNA-Seq data correlated well with qRT-PCR expression

194 analysis. The comparison c-Src(mt) vs. mock provided a comprehensive list of 434 c-Src(mt)
195 effector genes for further characterization.
196
197 **c-Src(mt) deregulates genes involved in migration, apoptosis and protein synthesis**
198 To gain further insights into the gene expression program mediated by c-Src(mt), we subjected
199 the set of c-Src(mt) effector genes to bioinformatic analyses. First, characteristics of gene
200 products were assessed through Gene Ontology (GO) overrepresentation analysis (ORA) [30].
201 Analysis of gene products falling into the GO category of 'Biological Process' (Figure 2A) showed
202 that c-Src(mt) effector genes play a prominent role in organelle organization (GO:0006996, q =
203 6.66×10^{-6}) (Figure 2A), indicating changes in cellular morphology. This is in line with the notion
204 that c-Src(mt) promotes an invasive phenotype in epithelial cells and impairs cell polarization
205 and cytoskeletal organization [17-20]. Moreover, expression of c-Src(mt) significantly altered
206 expression levels of genes involved in cellular responsiveness to cytokine stimulation
207 (GO:0034097, $q = 1.31 \times 10^{-3}$). It has been noted that the composition of cytokines produced in
208 tumor microenvironments play an important role in cellular migration [31]. For instance, c-
209 Src(mt)-expressing cells overexpress WNT5A ($q = 9.28 \times 10^{-3}$), a ligand of Frizzled receptors.
210 Wnt/Frizzled signaling is known to be implicated in cellular migration [32]. Apoptosis-associated
211 genes were deregulated in c-Src(mt)-expressing cells as well (GO:0006915, $q = 1.31 \times 10^{-3}$).
212 Evasion of apoptosis is recognized as a hallmark of cancer, and Src is known to play a key role in
213 this process [1]. Gene products of c-Src(mt) effector genes are mainly localized in the cytosol
214 and nucleus (Figure 2A, 'Cellular Component'), perhaps reflecting a predominant involvement in
215 cytosolic signaling and gene regulation processes. An enrichment of proteins binding to

structural proteins such as actin (GO:0003779, $q = 5.33 \times 10^{-4}$) indicate that c-Src(mt) effector genes are implicated in cytoskeleton remodeling and migration (Figure 2A, 'Molecular Function').

To further characterize the roles of c-Src(mt) effector genes, we performed pathway enrichment analysis using ConsensusPathDB [33]. We found that c-Src(mt) effector genes mainly affect pathways involved in cell migration and adhesion (Figure 2B, yellow), including hemidesmosome assembly and integrin pathways, as well as protein synthesis (Figure 2B, blue) and apoptosis (Figure 2B, red).

c-Src(mt) effector genes involved in cellular migration and adhesion comprise integrin receptor components *ITGA2*, *ITGA6* and *ITGAV*, all of which are downregulated (Figure 2C). Integrin receptors are known to attach cells to the extracellular matrix (ECM) by binding to ECM components such as laminin, collagen, fibronectin and E-cadherin, and their deregulation promotes invasiveness. For instance, *ITGAV* expression has been linked with metastatic activity in colorectal cancer [34], and *ITGA2* genetic variants may be associated with invasion of gastric cancer [35]. On the other hand, a diversity of components of the ECM itself was deregulated by the action of c-Src(mt). This includes upregulation of Decorin (*DCN*), Fibronectin 1 (*FN1*), Fibrillin 1 (*FBN1*), and downregulation of E-cadherin (*CDH1*) genes. Various genes encoding collagen chains are either up- (*COL8A1*, *COL12A1*, *COL28A1*) or downregulated (*COL17A1*). Of note, overexpression of Decorin [36], Fibronectin 1 [37] and collagen chains COL8A1 [38] and COL12A1 [39] have been recently shown to promote invasiveness of different cancer types. This indicates that c-Src(mt) significantly alters cell-matrix interactions, thereby decreasing cell adhesion and promoting cellular migration and invasiveness. Moreover, matrix

metalloproteinase-2 (MMP2), an enzyme degrading type IV collagen, the major constituent of basement membranes, is upregulated by c-Src(mt). Increased expression of MMP2 has been shown to correlate with cellular invasion [1]. Except for the collagen chain COL8A1 and Fibronectin 1, none of the above mentioned genes involved in cell adhesion are deregulated by c-Src(wt) (Figure 2C), further verifying that the activating mutation of c-Src(mt) promotes Src activity.

c-Src(mt) effector genes involved in apoptosis signaling include those involved in maintaining cytoskeleton integrity such as Adducin 1 (*ADD1*) and Plectin (*PLEC*), both of which were upregulated by c-Src(mt) (Figure 2C). Adducins are components important for the cortical cytoskeleton network and are subject to Caspase-3-mediated cleavage during cisplatin-mediated apoptosis [40]. Plectin serves a cross-linking molecule for the cytoplasmic filament system and is thus involved in maintaining cytoskeleton integrity, and its cleavage by Caspase-8 occurs early during TNF receptor-mediated apoptosis [41]. Upregulation of cytoskeletal components such as Adducin 1 and Plectin by c-Src(mt) might thus interfere with caspase-mediated cytoskeleton instability, thereby suppressing apoptosis. While *ADD1* was not deregulated by c-Src(wt), *PLEC* was upregulated, however, less markedly compared with c-Src(mt) (Figure 2C).

While the involvement of Src in cell adhesion and apoptosis is well-known [1], we also found a surprisingly large number of pathways involved in translation and protein synthesis with an overrepresentation of c-Src(mt) effector genes (Figure 2B). These include various steps of protein synthesis, such as mRNA transport and degradation, translation initiation, as well as amino acid biosynthesis and transport (Additional file 3). A number of genes encoding

eukaryotic translation initiation factors (EIFs), including *EIF3A*, *EIF3E*, *EIF4A2* and *EIF4G1* and other proteins involved in translation initiation were upregulated by c-Src(mt) (Figure 2C). Genes for small and large subunit ribosomal proteins, *RPL29*, *RPL31* and *RPS24*, were also upregulated. Amino acid transport across the cell membrane appeared to be heavily influenced by c-Src(mt) through deregulation of a number of genes encoding amino acid-transporting solute carrier proteins (SLCs), such as *SLC1A5*, *SLC3A2*, *SLC7A2*, *SLC7A5* and *SLC38A1*. In addition, amino acid biosynthesis was regulated by c-Src(mt), including downregulation of genes for serine hydroxymethyltransferases 1 and 2 (*SHMT1/2*), enzymes that catalyze the conversion of serine to glycine, and downregulation of the glycine dehydrogenase (decarboxylating) gene (*GLDC*) whose gene product degrades glycine. We propose that c-Src(mt) stimulates global protein synthesis by increasing the number of ribosomes and the intracellular levels of translation initiation factors and free amino acids. Moreover, genes involved in mRNA decay, such as the *CNOT6* cytoplasmic deadenylase, were downregulated by c-Src(mt), while the polyadenylate-binding protein 1 (*PABPC1*) gene, essential for translation initiation, was upregulated. Moreover, the overall higher proportion of upregulated compared to downregulated genes on the transcriptional level induced by the expression of c-Src(mt) indicated an elevated pool of available mRNAs, further increasing protein expression, including that of pro-invasion and anti-apoptosis genes (Figure 2A-C). Of note, only few of the above mentioned genes involved in protein synthesis were deregulated by c-Src(wt) (Figure 2C). We conclude that the activation of protein synthesis requires the constitutively active form of c-Src, c-Src(mt).

281 In summary, physiological expression of c-Src(mt) was sufficient to modulate cell
282 migration/adhesion, apoptosis and protein synthesis mainly through deregulation of genes
283 involved in integrin signaling, ECM remodeling, composition of the cytoskeleton and of the
284 translation machinery.
285
286 **c-Src(mt) promotes tissue invasion *in vivo***
287 Elevated Src activity frequently correlates with increased tissue invasion activity [42]. The
288 change in expression levels of migration/adhesion-associated genes in the c-Src(mt)-expressing
289 cells (Figure 2B-C) suggested that these cells might also bear increased invasive potential. To
290 test whether c-Src(mt)-expressing cells exhibit increased tissue invasion *in vivo*, we employed an
291 established mouse model of lung tissue invasion [43]. 8-weeks old SCID mice were intravenously
292 injected with 9×10^5 naïve MCF-10A, c-Src(wt), c-Src(mt), or mock-expressing cells. To induce
293 transgene expression in the engineered cell lines, 1 µg/mL Tet was added to the drinking water
294 of all animals. Mice were sacrificed after 42 days, and lung tissues analyzed phenotypically
295 (Figure 3A). Macroscopic analysis revealed visible lung metastases exclusively in those mice that
296 have received c-Src(mt)-expressing cells, indicating that expression of c-Src(mt) predisposes
297 tumor cells to extravasate and migrate into epithelial tissue. Lung metastases were found in two
298 of five mice injected i.v. with c-Src(mt)-expressing cells, while they were absent in the lungs of
299 mice that received c-Src(wt)-expressing cells (0/4) or mock and naïve MCF-10A cells (both 0/5)
300 (Figure 3B). Representative histological H&E-stained sections are shown in Figure 3C.
301 Subsequent histological analyses corroborated the presence of metastases in the macroscopic
302 positive lungs of mice that have received c-Src(mt)-expressing cells (Figure 3D).

303 Immunohistological analyses indicated proliferating tumor cells mainly at the outer radial
304 boarder of the lung metastases (Ki67 in Figure 3D), with little necrosis (Cleaved Caspase 3). Only
305 a few F4/80$^+$ macrophages, Ly6G$^+$ granulocytes, CD3$^+$ T cells as well as B220$^+$ B cells were found
306 at the boarder of the metastases, indicating the failure of the murine immune system to attack
307 tissue-invading cells.
308 The ability of c-Src(mt)-expressing cells to invade lung tissue in mice verified the expression
309 signature that contained migration/adhesion-associated genes (Figure 2B-C) and is in
310 accordance with previous findings that cells expressing this c-Src mutant exhibit increased
311 motility in *in vitro* migration assays [19]. In contrast, we could not detect any tissue invasion in
312 mice that received c-Src(wt)-expressing cells. This might well be due to the fact that most of the
313 migration/adhesion-associated genes that were deregulated by c-Src(mt) were not altered by c-
314 Src(wt) (Figure 2C). This is in accordance with the fact that overexpression of the wildtype c-Src
315 protein alone causes only minimal changes in the migratory activity [19,29,44], which may be
316 explained by interactions with PDZ domain-containing tumor suppressors with the C-terminal
317 GENL motif of the c-Src(wt) protein that suppress its kinase activity [17,18,20]. This is also
318 corroborated by the observation that c-Src(mt)- but not c-Src(wt)-expressing cells exhibited a
319 transformed phenotype upon stimulation with Tet in cell culture (data not shown).
320
321 **Non-migration-associated c-Src(mt) effector genes are involved in mRNA transport,**
322 **translation and iron transport**
323 The role of activated c-Src in promoting metastasis is well-known [1]. However, our
324 transcriptome data also revealed a prominent effect of c-Src(mt)-expression on protein

synthesis (Figure 2B). To identify c-Src(mt) effectors that are not associated with metastasis, we employed a literature mining approach in PubMed abstracts, using a defined lexicon of terms associated with migration. The majority of c-Src(mt) effector genes, 415 of 434 (95.6%) were found in PubMed abstracts. Of these, 344 (83%) were significantly linked to migration, which might reflect that previous studies investigating Src activity were biased towards its migration-promoting activity. On the other hand, 71 genes (17%) were not linked to migration (highlighted green in Additional file 1: Table S2). These 71 genes are of particular interest as they might contain genes that have previously not been associated with Src activity. To get an idea about the cellular processes these 71 genes are implicated in, they were subjected to GO overrepresentation analysis. Interestingly, these genes appeared to be mainly involved in (m)RNA transport and splicing (Figure 4). This corroborates our finding that c-Src(mt) exerts a previously underestimated role in the regulation of gene expression at the level of translation (Figure 2B-C). Our data so far suggests that the constitutively active c-Src(mt) mutant, in addition to promoting metastasis, may have an impact on protein synthesis. This function, to our knowledge, has not yet been attributed to Src activity.

c-Src(mt)-expressing cells exhibit characteristics of primary human tumors and progression to metastasis

Increased Src activity is a frequent feature of different human cancers [1]. Therefore, we wondered whether c-Src(mt)-expressing cells show similarities with primary human cancer cells. To address if c-Src(mt)-expressing cells share similar gene expression profiles with primary human cancers, we used the ONCOMINE cancer microarray database [45] for comparison with

c-Src(mt) effector genes. The gene sets in ONCOMINE consists of genes that were found differentially expressed when comparing either tumor vs. normal tissue or metastatic vs. primary tumors and are provided as gene sets within the database. 38 of 70 (54%) of the ONCOMINE gene sets of normal tissue vs. cancer pairs showed significant ($q \leq 0.05$) overlap (Figure 5A, left). Of note, this included most of the breast cancer (4/6), leukemia (5/6), ovarian (5/7), prostate (7/13) and renal cancers (3/5), as well as all liver cancer samples (4/4). Increased Src activity has been identified in breast, ovarian, liver cancer and leukemia [42], as well as prostate [46] and renal cancer [47]. Analysis of ONCOMINE datasets of cancer progression (metastatic vs. localized, n=42) revealed that 18 of 42 (40%) of these gene sets had significant overlap with c-Src(mt) effector genes (Figure 5A, right), including most of the lung (4/5) and the prostate (9/16) cancer samples. Increased Src activity has been shown to be associated with increased metastasis, amongst others, in lung [42] and prostate cancer [48]. Therefore, c-Src(mt)-expressing cells proved to be a valid, albeit simplified, model for some human cancers and their progression to metastasis. This suggests that the single amino acid mutation of c-Src(mt) can indeed induce a 'cancer-like' state of the cell, in the sense that it affects similar genes found to be dysregulated in diverse tumor or metastatic cells.

We next sought to investigate whether the non-migration-associated c-Src(mt) effector genes (Additional file 1: Table S2) in particular are relevant for human cancer. Therefore, we searched the ONCOMINE database for any evidence of their deregulation in normal tissue vs. cancer pairs. We identified 18 genes that showed either significant ($q \leq 0.05$) up- (eight genes) or downregulation (ten genes) in at least 50% of the respective cancer types (Figure 5B) similar to our cellular model, in which mock cells represent normal tissue and c-Src(mt)-expressing cells

correspond to cancer cells. Of note, most of these genes were deregulated in leukemia samples. Thus, the non-migration-associated c-Src(mt) effector genes are likely most relevant for this cancer type. This might reflect the fact that the absence of the PDZ binding motif at the C-terminus of c-Src(mt) is reminiscent of other SFK members, such as Fgr or Hck (Figure 1A) expressed in non-adherent cells of the hematopoietic system.

Discussion

Here we have analyzed a cellular model of C-terminally mutant and constitutively active c-Src(mt) by its expression at physiological levels in non-transformed human cells. Of note, the expression of c-Src(mt), but not of c-Src(wt) at comparable levels (Figure 1C), altered the expression of previously reported Src targets (Additional file 1: Figure S4). This is in accordance with previous findings that activation of c-Src, either by activating mutations or deletions in the C-terminus [17-20,24], or *via* the action of co-factors, such as FAK [1], is required for its transforming activity. Whole-genome transcriptome analysis revealed that most of the 434 genes deregulated by c-Src(mt) are implicated in migration and adhesion, apoptosis and protein synthesis (Figure 2A-C). In accordance with transcriptome data and previous findings, c-Src(mt)-expressing cells exhibited increased invasion of lung tissue and metastasis formation *in vivo* after i.v. injection in mice (Figure 3A-D). In contrast, c-Src(wt)-expressing cells did not induce metastases in this animal model, reflecting that expression levels of most genes relevant for migration/adhesion that were deregulated by c-Src(mt) were not altered by c-Src(wt). The herein investigated c-Src proteins, c-Src(wt) and c-Src(mt), only differ by a single amino acid at

the very C-terminus (Figure 1A). The C-terminal GENL sequence of c-Src is a known binding motif for PDZ domain-containing tumor suppressor proteins [17-20]. Binding of these tumor suppressors keeps c-Src in an inactivated state, which may explain why expression of c-Src(wt) with the intact GENL motif, in contrast to c-Src(mt) with the C-terminal GENA sequence, was insufficient to alter the expression of known Src targets (Additional file 1: Figure S4) or to induce metastatic behavior (Figure 3A-D). The *in vivo* data shown here is in agreement with previous findings showing that c-Src(mt)-expressing cells exhibits increased migration activity compared to c-Src(wt)-expressing cells *in vitro* [17-20].

Literature mining revealed that more than 80% of c-Src(mt) effector genes have previously been linked to cellular migration. The remaining 17% of genes showed an enrichment genes involved in (m)RNA transport and splicing processes (Figure 4), which are relevant for gene expression at the level of translation. Interestingly, various cellular pathways relevant for protein synthesis showed an enrichment of genes deregulated by c-Src(mt) (Figure 2B). To our knowledge, the deregulation of protein synthesis has not yet been linked with Src activity, perhaps because most previous studies were mainly focused on investigating its metastasis-promoting potential. Therefore, we suggest that Src activity is associated with an increase in global protein synthesis. Interestingly, this function has recently been attributed to another proto-oncogene, c-Myc [49]. The importance of protein synthesis in cancer development has been neglected historically [49]. However, recent evidence underlines the importance of deregulated translation in different cellular processes, including cancer progression. Using ribosomal profiling, Hsieh et al. [50] have shown that oncogenic signaling of the mammalian target of rapamycin (mTOR) kinase heavily influences the translational machinery, and thereby cancer invasion and metastasis, through

increased translation of pro-invasion mRNAs. Consequently, components of the translational machinery, such as the translation initiation factor EIF4E, are now being explored as therapeutic targets to treat cancer [49]. In line with these findings, we found evidence that Src activity causes the deregulation of different steps of the translational control. Proteins that are upregulated by c-Src(mt) include those acting as translation initiation factors, ribosomal proteins and those promoting mRNA stability, thereby potentially promoting the 'cancerous' translation machinery. Interestingly, Src is known to activate phosphoinositide 3-kinase (PI3K) by phosphorylation, whose downstream targets include mTOR [49]. mTOR stimulates protein synthesis by phosphorylating EIF4EBP1 and ribosomal S6 kinase p70S6K1/2. Consequently, mTOR and downstream targets p70S6K1/2, EIF4EBP1 and ribosomal protein 6 are phosphorylated/activated by Src [49]. We therefore propose a dual mechanism of translational activation by Src, through cytosolic activation of the mTOR pathway and transcriptional activation of components of the translational machinery, as shown in this study. This may lead to increased translation of pro-invasive and anti-apoptotic mRNAs, thus potentially promoting cellular migration.

Another potential target for cancer therapies is FAK, a cytoplasmic tyrosine kinase that is a key downstream signal transducer of integrin receptors [51,52]. FAK is directly phosphorylated by Src kinase, leading to the activation of multiple intracellular signaling pathways that are relevant, amongst others, for apoptosis and cell migration [52]. FAK and Src kinase synergize in cell migration and invasion [53,54]. We have recently shown that the expression of c-Src(mt) leads to increased levels of phosphorylated FAK in MCF-10A cells concomitant with elevated cell motility [19]. The transcriptome analysis of this study revealed that the mRNA expression level

of FAK was not significantly influenced by c-Src(mt), as FAK (gene symbol: *PTK2* for Protein Tyrosine Kinase 2) was not among the c-Src effector genes (Additional file 1: Table S2). Together with our previous findings [19] the results here indicate that FAK activation by c-Src(mt) mainly involves its Src-dependent phosphorylation while transcription levels remain unaltered. Of note, both upregulation and increased phosphorylation of FAK have been detected in numerous human metastatic tumors compared to benign, non-metastatic tumors or normal tissues [52]. For instance, increased FAK phosphorylation has been identified in primary breast cancer specimens and breast cancer cell lines compared to normal tissue [55,56].

In line with these findings, the gene expression changes induced by c-Src(mt) showed significant similarities with expression profiles of many primary human cancers, especially breast cancer, leukemia, ovarian, prostate and renal cancers (Figure 5A). Of note, we detected significant overlap with expression profiles of some colon cancers (3/6 tumor vs. normal tissue and 1/3 metastatic vs. localized), for which activating C-terminal mutations of the c-Src gene have been reported before [24]. Thus, c-Src(mt)-expressing cells not only exerted increased migratory potential, but also showed similarities to the gene expression signature of some primary cancer disease, which may become metastatic later. It has to be noted that tumorigenesis and progression to metastasis *in vivo* is a multi-step process that involves the inactivation of tumor suppressor genes and the acquisition of oncogenic mutations [57,58]. This study aimed to characterize the function and effectors of activated Src kinase with loss of binding to PDZ domains and not mechanisms of cancer formation or metastasis in general.

Previous genome-wide analyses of the effect of Src activity on gene transcription include microarray analyses of v-Src-transformed mouse cells [3,59] or RSV-transformed chicken cells

[60,61]. One study analyzed the gene transcription profile of MCF-10A cells transformed with ER-Src, a derivative of v-Src that is fused to the ligand-binding domain of the estrogen receptor [7]. In contrast, the cellular model described herein reduces the metastatic mechanism to a single point mutation in the c-Src protein and potential escape from tumor suppressors, leading to increased kinase activity. This might explain why there is only little concordance with the c-Src(mt) effector genes identified in this study and the v-Src-regulated genes identified by others [3,7,59-61]. Other more complicated multi-component mechanisms certainly exist but may be difficult to analyze.

In addition, we identified 71 c-Src(mt) effector genes that have not been previously associated with metastasis, which were mainly involved in the processes of RNA transport and splicing, and thus influence protein synthesis (Figure 4). A number of these genes showed deregulation in human primary cancer samples as well, especially in leukemia (Figure 5B). SFK members lacking the ability to bind to PDZ proteins, such as Fgr or Hck, are typical for non-adherent hematopoietic cells. Thus, non-adherent hematopoietic cells resemble metastatic alterations of adherent cells to some degree.

Conclusions

This study first defines the regulatory landscape of a specific C-terminally mutated form of the c-Src kinase, c-Src(mt). Cells expressing this mutant extravasated and migrated into lung tissue of mice *in vivo*, thus mimicking metastasizing cells. Among known genes, we describe a number of genes not yet associated with this behavior. They might deserve further investigation as

putative biomarkers of invasive cells or therapeutic approaches especially in the context of Src-driven progression to metastasis.

Methods

Cells

MCF-10A cells (ATCC-CRL-10317) were obtained from the American Type Culture Collection and grown in complete growth medium [DMEM/F12 (Gibco) with 2 mM L-glutamine supplemented with 20 ng/mL epidermal growth factor (Sigma), 100 ng/mL cholera toxin (Sigma), 10 ng/mL insulin (Sigma), 500 ng/mL hydrocortisone (Sigma) and 5% horse serum (Gibco)]. Cells were maintained at 37°C with 5% CO_2.

Lentiviral constructs

The HA-tagged Src constructs are described elsewhere [17-19]. Lentiviral particles for TetR-inducible expression of c-Src(wt) (LVP-Src-wt) and c-Src(mt) (LVP-Src-mt), as well as a short nonsense construct (LVP-mock) conferred resistance to Blasticidin S. LVP017-Neo allows constitutive expression of TetR and confers resistance to G418 (Additional file 1: Figure S1). Lentiviral particles were prepared by AMS Biotechnology (United Kingdom). Sequences were confirmed by capillary sequencing.

Generation of stably expressing cells

MCF-10A cells were grown to 50% confluence in complete growth medium. Cells were incubated with LVP017-Neo at a multiplicity of infection (MOI) of ten and lentiviral expression particles (MOI = 3) for 72h. Then, medium was replaced with fresh complete growth medium

498 containing 60 μg/mL G418 (Gibco) and 3.5 μg/mL Blasticidin S (Invitrogen) for 14 days to select
499 double-positive cells. Untransfected control cells were killed within 14 days when exposed to
500 either 60 μg/mL G418 or 3.5 μg/mL Bsd. Double-positive cells were maintained in complete
501 growth medium with 30 μg/mL G418 and 1.75 μg/mL Bsd. Expression of TetR mRNA was
502 confirmed by qRT-PCR and was at comparable levels in the three engineered cell lines. The
503 tetracycline-inducible system allowed regulation of transgene expression by adding tetracycline
504 (Tet) to the cell culture medium. Inducible expression of the transgene in c-Src(wt)- and c-
505 Src(mt)-transfected cells was verified by qRT-PCR and Western Blot. For the subsequent gene
506 expression analyses we selected cell populations that showed similar levels of c-Src(wt) and c-
507 Src(mt) expression at physiological levels (Figure 1C).

508 **Preparation of total RNA**

509 Cells were grown to ~70% confluence in complete medium supplemented with 30 μg/mL G418
510 and 1.75 μg/mL Bsd. Tetracycline (Sigma-Aldrich) was added to the culture medium and cells
511 were incubated for 24h. Then, total RNA was extracted with Trizol (Invitrogen) according to the
512 manufacturer's recommendations. The RNA was precipitated at -80°C and pelleted by
513 centrifugation at 12,000 x g for 10 minutes. The pellets were washed with 75% ethanol, air-
514 dried and solubilized in nuclease-free water. Remaining DNA was removed with the RNase-free
515 DNase set from Qiagen according to the manufacturer's protocol. Then, the solution was
516 subjected to phenol-chloroform extraction. RNA integrity was assessed by agarose gel
517 electrophoresis.

518 **Primer design and synthesis**

519 Primers for quantitative Real-Time PCR (qRT-PCR) were designed such that PCR products were
520 smaller than 300 bp and intron-spanning, using the primer3 program
521 (http://primer3.wi.mit.edu) [62] and cDNA sequences retrieved from the UCSC Genome
522 Browser and the current release of the human genome [63]. The GAPDH primer pair has been
523 described previously [64]. The forward primer for mu SRC_HA is situated within the HA tag of c-
524 Src constructs, c-Src(wt) and c-Src(mt), to avoid amplification of endogenous c-Src expressed by
525 MCF-10A cells. Primers were synthesized by Metabion (Martinsried, Germany). For a complete
526 list of primer sequences used in this study refer to Additional file 1: Table S1.

527 **Quantitative real-time PCR**

528 Total RNA preparations were reverse-transcribed with the High Capacity cDNA Reverse
529 Transcription Kit (Applied Biosciences) according to the manufacturer's recommendations. RT-
530 products were quantified with the Fast SYBR Green Master Mix (Applied Biosystems) on an
531 Applied Biosystems 7900HT Fast Real-Time PCR System. Cycle conditions were: 10 min.
532 incubation at 95°C, followed by 50 amplification cycles of 95°C for 15 s and 58°C for 1 min.

533 **Western Blots**

534 Cells were resuspended in buffer A (10 mM HEPES, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM
535 DTT, protease inhibitor cocktail) and layzed mechanically. After centrifugation for 5 min at 2500
536 x g the pellet then was subjected to DNase I digestion (10 mM HEPES, pH 7.4, 10 mM NaCl, 3
537 mM $MgCl_2$, 0.2U DNase I, 1 mM DTT, protease inhibitor cocktail) and centrifuged again as
538 described before. The resulting supernatant was combined with the first one and the total
539 protein concentration determined using the Bradford method (Sigma-Aldrich) following the
540 manufacturer's instructions. 30 µg of lysate were subjected to SDS-polyacrylamide gel

electrophoresis (PAGE) and immunoblotting. Primary antibodies used in the experiment were: anti-Src clone GD11 (Millipore), and anti-GAPDH (Ambion #AM4300). The secondary antibody was Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch).

RNA sequencing

1 µg of total RNA preparations of the four cell lines were subjected to cDNA library preparation with the NEBNext Ultra RNA Library Prep Kit (New England Biolabs) and subsequently to DNA sequencing on an Illumina Genome Analyzer IIx sequencer with a 100 bp paired-end multiplex run, yielding 99,100,500 (naïve MCF-10A), 46,988,060 (mock), 38,071,856 (c-Src(wt)) and 56,106,530 (c-Src(mt)) quality control-passed reads.

Sequence read alignment

The Illumina paired-end sequence reads were mapped to the UCSC hg19 human reference genome using the Bowtie read aligner (0.12.8 release, default parameters) [21].

Gene expression and determination of differential expression

Via the Illumina RNAseq technology, gene expression in four different cell lines was measured:

(1) non-transfected cells (naïve MCF-10A)

(2) cells transfected with the mock lentiviral vector

(3) cells transfected with the c-Src(wt) lentiviral vector

(4) cells transfected with the c-Src(mt) lentiviral vector

RNA expression levels were determined with a standardized analysis pipeline based on RSEM (RNA-Seq by Expectation Maximization) [22]. DESeq [23] was then used to determine differential expression of genes comparing different cell lines. Genes with an FDR-adjusted q value ≤ 0.05 in their fold change were considered as differentially expressed.

Gene Ontology overrepresentation

The ConsensusPathDB [33] was used to identify predominantly occurring GO terms among the candidate genes in order to determine functional commonalities between them. p values were derived from a Fischer's exact test performed for the candidate genes against the background set of all genes in the human genome. Correction for multiple testing was done using the FDR (false discovery rate) method that resulted in q values.

Pathway overrepresentation

Overrepresented pathways involving the candidate genes were accumulated *via* the interface of the ConsensusPathDB (Release 25) [33], a pathway database that integrates interaction data from currently 31 public resources. p and q values were calculated analogously to the above GO analysis.

Mouse experiments

All animal procedures were done in accordance with the regulations of the Cantonal Veterinary Authority of Zurich and the Swiss laws on animal protection. For the tumor growth studies, severe combined immune-deficient (SCID) mice were obtained from Charles River laboratories. Eight-week-old female mice were intravenously injected with $9x10^5$ MCF-10A cells or c-Src(wt), c-Src(mt) or mock cells. Lung tissues were evaluated after 42 days. 1 µg/mL of Tet was added to the drinking water of all mice to induce transgene expression in engineered cell lines.

Immunohistochemistry

Lung tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin sections (2 µm) were either stained with haematoxylin / eosin (H&E) or automated immunohistochemistry staining with rabbit-anti-CD3 for T cells, rat-anti-B220 for B cells (both

BD Biosciences), rat-anti-F4/80 for macrophages (BMA Biomedicals AG), rat-anti-Ly6G for granulocytes (BD Pharmingen), rabbit-anti-Ki67 for proliferating cells (Neomarkers), rabbit-anti-cleaved caspase 3 for apoptotic cells (Cell Signaling, Danvers, USA), rabbit-anti-rat HRP and anti-rabbit-poly HRP (Leica Bond Polymer Refine Detection DS9800). Image acquisition was performed on DotSlide BX51 (Olympus), SCN400 (Leica), Axio Z1 (Zeiss) or BX53 (Olympus) microscopes.

Literature mining

We aimed to find genes that can be associated to cellular migration characteristics but have so far not been dealt with in that context. Thus, we used an automated literature mining approach to scan PubMed abstracts for information on the candidate genes with respect to migration. For that purpose we defined a lexicon of migration-related terms (migration, metastasis, pseudopodia, wound healing, adherence, adhesion, homing, pathfinding, motility, angiogenesis, invasion, cancer progression, PDZ domain, cytoskeleton) and searched for the genes' co-occurrence with those terms. We used an evidence score defined as

$$s_{ij} = \log_2(P_{ij} / (P_i P_j))$$

to quantify literature evidence for a gene.

Here, P_{ij} is the frequency of co-occurrence of the lexicon term j and the gene j and P_i and P_j are their marginal frequencies. The evidence score for each gene was computed as the sum of co-associations over the entire lexicon

$$s_j = \Sigma_i s_{ij} .$$

A high score indicates that a gene has often been related to metastasis and other terms of the lexicon. It is thus of particular interest to further analyze those genes that have a low or negative evidence score. 71 genes showed no evidence.

Cancer gene expression profiles

Gene set enrichment analysis was performed on the basis of publicly available cancer gene expression datasets from the ONCOMINE database [45]. We looked for cancer datasets that showed similar expression profiles to our c-Src(mt)-expressing cells vs. mock cells. Focus was laid on studies that compared different stages of cancer tissue, i.e. sets marked with NP for 'normal tissue vs. cancer' and CP for 'cancer progression vs. primary tumor'. At the state of writing there were 112 of such data sets to be found, all of which were considered. Ideally, those sets containing data for normal cancer tissue as well as metastatic tissue show a significant overlap of differentially expressed genes when compared to our set of c-Src(mt) effector genes. To calculate the significance of overlap of our effector genes with the gene sets from the ONCOMINE data sets, we performed Fischer's exact test and corrected for multiple testing using the FDR method.

Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional files.

Additional files

Additional file 1: Figures S1-S5 and Tables S1 and S2.

Additional file 2: Gene Ontology Overrepresentation Analysis of c-Src(mt) effector genes.

Additional file 3: Pathway Overrepresentation Analysis of c-Src(mt) effector genes.

Additional file 4: Gene Ontology Overrepresentation Analysis of non-metastasis-associated c-Src(mt) effector genes.

Additional file 5: ONCOMINE analysis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FB performed the *in vitro* experiments, CH and RH conducted the bioinformatic evaluations, BT and MK performed Illumina sequencing, AW and MRS prepared RNA for sequencing and performed Western Blots, LB and MH performed and interpreted mouse studies, HL and KM designed and initiated the study. FB and KM wrote the manuscript. All authors read and approved the final manuscripts.

Acknowledgements

We like to thank Dr. Jochen Heinrich (University of Zurich) for help with generating the c-Src expression constructs, Nada Kumer (Max Planck Institute for molecular Genetics) for excellent

technical assistance, Dr. Algirdas Ziogas (University Hospital Zurich) for help with some of the experiments, and Profs. Peter H. Seeberger (Max Planck Institute of Colloids and Interfaces) and Martin Vingron (Max Planck Institute for molecular Genetics) for their generous support. We thank the BMBF (grants 0315428A and 0316190A to RH) and the Max Planck Society for financial support. This work was supported by private funding (KM). MH was supported by the Helmholtz Alliance PCCC, an ERC Starting grant (LiverCancerMechanism), the Helmholtz Foundation, the SFBTR36 and the Hofschneider Foundation. The authors declare no conflicts of interest.

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Figure Legends

Figure 1 Establishment of a cellular model of activated c-Src. (A) Alignment of C-terminal moieties of human Src family kinases (SFKs). A conserved binding motif for PDZ domain-containing tumor suppressor proteins is shown green. **(B)** Domain organization of c-Src constructs used in this study. **(C)** Detection of transgene expression in engineered MCF-10A cells by Western Blot (upper part) and qRT-PCR (lower part). The α-Src antibody binds to both human (hu) and murine (mu) c-Src proteins. The mu c-Src constructs (61.3 kD) are bigger than the endogenous hu c-Src (59.8 kDa), giving rise to a second band above that of hu c-Src in the c-Src(wt) and c-Src(mt)-expressing cells. GAPDH runs at 36 kDa. qRT-PCR data shows mean + SD of two independent measurements. **(D)** Density plot showing logFC values of statistically significant ($q \leq 0.05$) DE genes of mock, c-Src(wt) and c-Src(mt) cells compared with naïve MCF-10A, obtained from RNA sequencing. **(E)** Correlation between RNA sequencing data and qRT-

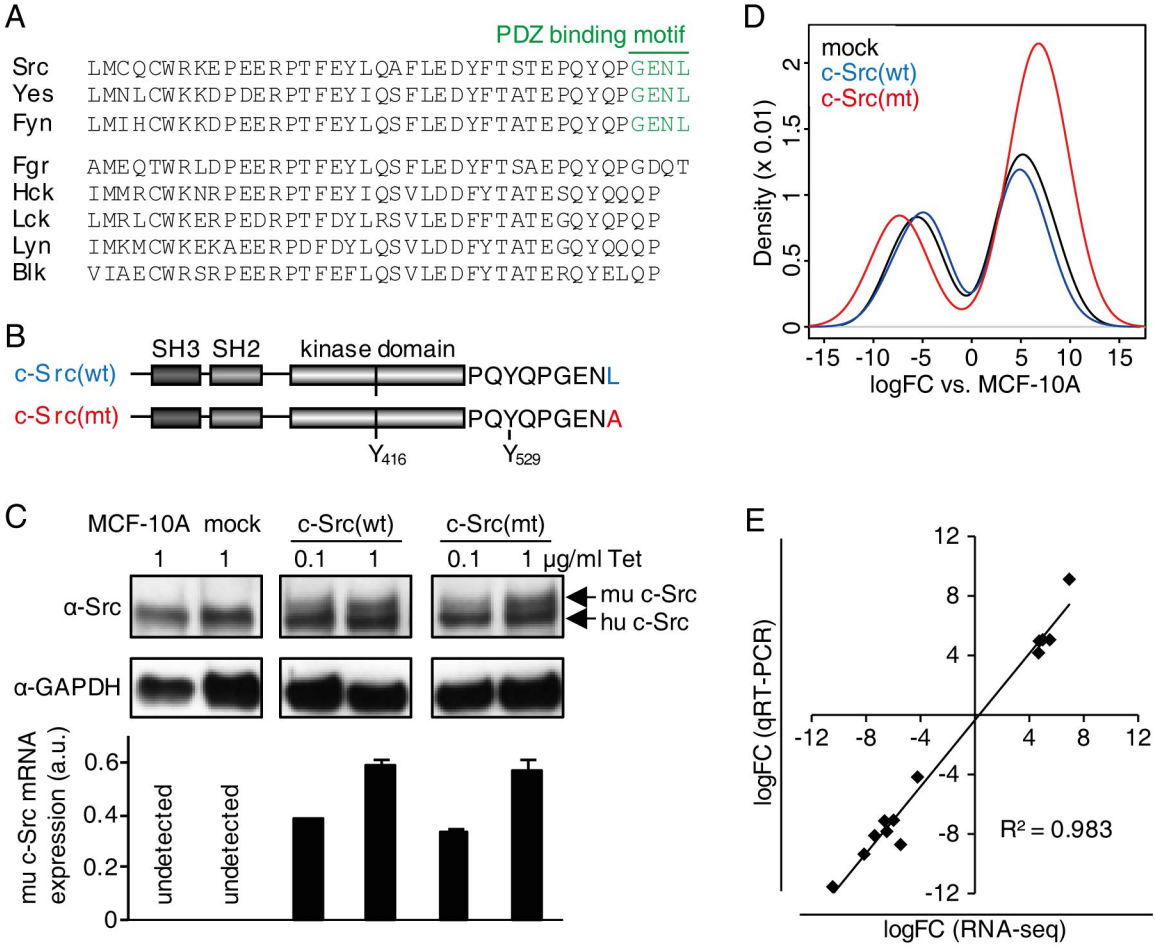
PCR measurements of 13 statistically significantly DE genes of the comparison c-Src(mt) vs. mock cells (Additional file 1: Figure S5).

Figure 2 Functional analysis of c-Src(mt) effector genes. (A) Gene Ontology (GO) enrichment analysis. Displayed are the ten level 4 GO terms in the indicated categories with the lowest q values (see Additional file 2 for a complete list and corresponding gene lists). **(B)** Pathway analysis. Displayed are the twenty pathways with the lowest q values. Redundant pathways have been removed. Color code indicates pathways associated with different cellular processes. The complete list of pathways can be found in Additional file 3. **(C)** Selected genes and their relative expression levels in c-Src(mt) and c-Src(wt)-expressing cells with respect to mock cells. logFC values ($q \leq 0.05$) are indicated by color code. Non-significant ($q > 0.05$) gene expression is shown in grey.

Figure 3 c-Src(mt)-expressing cells exhibit elevated invasive potential *in vivo*. (A) SCID mice were injected i.v. with one of the four different cell lines and sacrificed 42 days later for histological analyses of their lungs. **(B)** Determination of lung metastases in mice injected with c-Src(mt)-expressing cells as visualized by H&E (Hematoxylin & Eosin) staining shown in C. **(C)** Macroscopic analysis of lungs. The arrow indicates a representative metastasis found in a mouse injected with c-Src(mt)-expressing cells. **(D)** Immunohistological stainings of lung tissues. Ki67, proliferating cells; Cl. Casp 3, apoptotic cells; F4/80, macrophages; Ly6G, granulocytes; CD3, T cells; B220, B cells. Scale bars, 50 μm.

Figure 4 Gene Ontology overrepresentation analysis of c-Src(mt) effector genes not linked to migration. For details, refer to Additional file 4.

Figure 5 ONCOMINE analysis of c-Src(mt) effector genes. (A) q values of gene expression overlaps with cancer vs. normal tissue pairs (left) and with metastatic vs. localized cancer pairs (left) are shown. A complete list of primary cancer samples and gene lists can be found in Additional file 5. **(B)** Non-migration-associated c-Src(mt) effector genes with significant deregulation in cancer vs. normal tissue pairs are shown.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Fgr AMEQTWRLDPEERPTFEYLQSFLEDYFTSAEPQYQPGDQT
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Lck LMRLCWKERPEDRPTFDYLRSVLEDFFTATEGQYQPQP
Lyn IMKMCWKEKAEERPDFDYLQSVLDDFYTATEGQYQQQP
Blk VIAECWRSRPEERPTFEFLQSVLEDFYTATERQYELQP
B
SH3 SH2 kinase domain
c-Src(wt) PQYQPGENL
c-Src(mt) PQYQPGENA
Y416
Y529
C
MCF-10A mock c-Src(wt) c-Src(mt)
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α-Src
mu c-Src
hu c-Src
α-GAPDH
mu c-Src mRNA expression (a.u.)
0.6
0.4
0.2
0
undetected
undetected
D
mock
c-Src(wt)
c-Src(mt)
Density (x 0.01)
0
0.5
1
1.5
2
-15 -10 -5 0 5 10 15
logFC vs. MCF-10A
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logFC (qRT-PCR)
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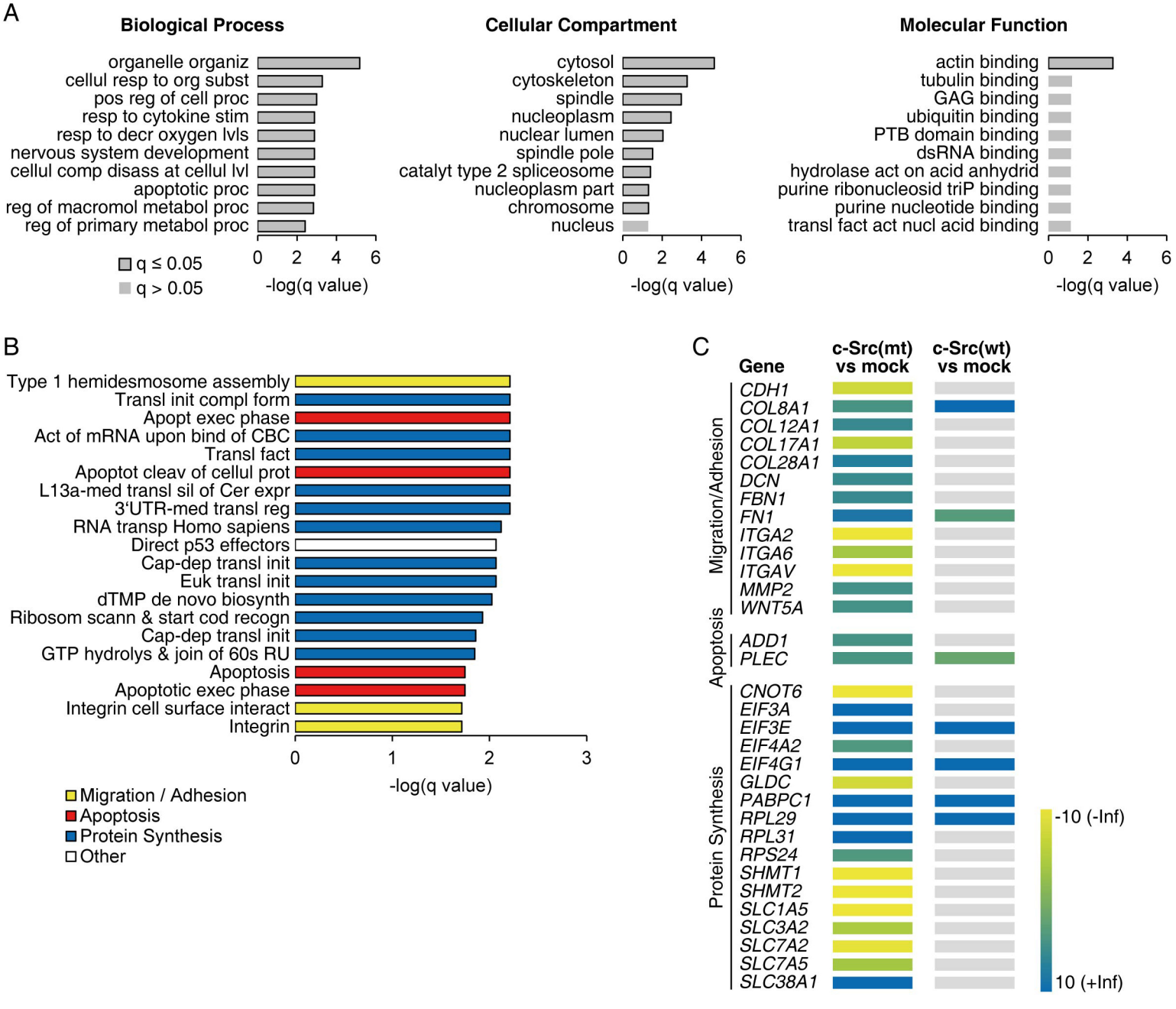
8
4
-4
-8
-12
-12 -8 -4 4 8 12
R² = 0.983
logFC (RNA-seq)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apoptotic proc
reg of macromol metabol proc
reg of primary metabol proc
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q > 0.05
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Direct p53 effectors
Cap-dep transl init
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dTMP de novo biosynth
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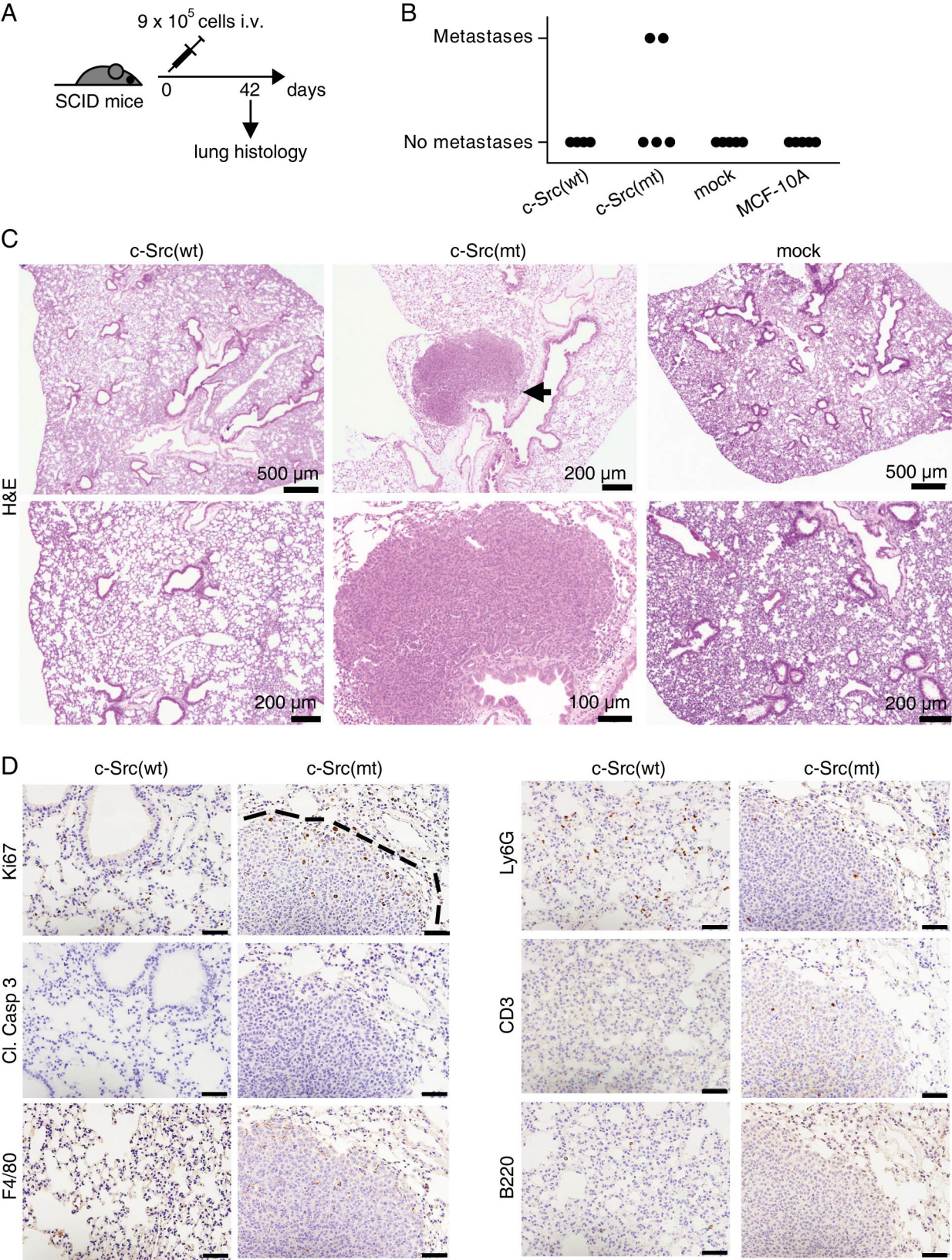
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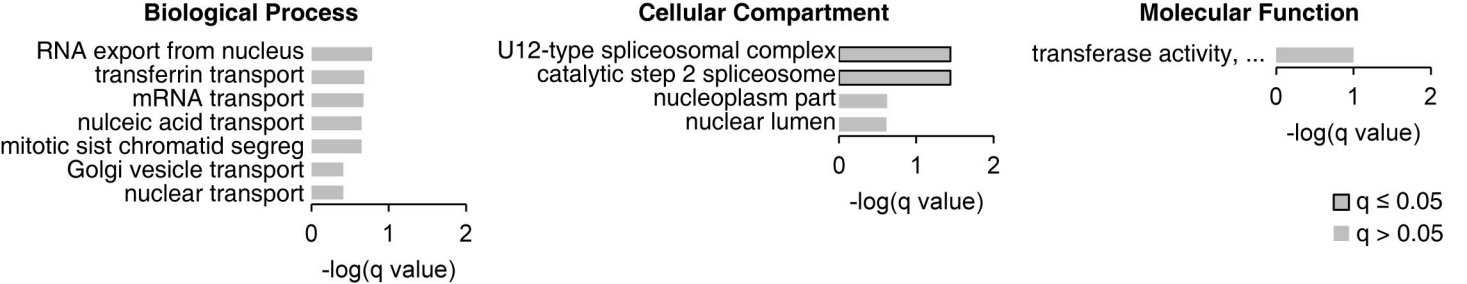
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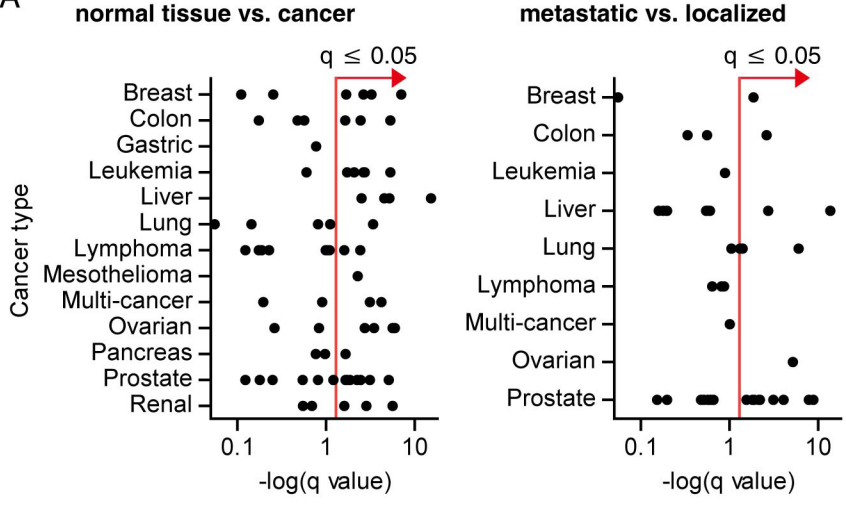

B

Cancer	Gene	Over-expressed	Under-expressed
Leukemia	*CDC40*	-	5 of 6
	MARK3	-	4 of 6
	PSRC1	-	2 of 3
	SMCHD1	-	4 of 6
	TTC7A	3 of 3	-
	ZNF655	3 of 3	-
Liver	*SHMT2*	-	4 of 4
Lung	*UACA*	-	2 of 3
Lymphoma	*IQCE*	6 of 8	-
Multicancer	*ANKS1B*	2 of 2	-
	KIAA0664	-	2 of 2
	THSD4	-	2 of 2
	ZNF451	2 of 2	-
Ovarian	*ASXL2*	4 of 5	-
	CDC40	-	4 of 5
	DHX15	-	4 of 5
	TNPO2	3 of 5	-
	YTHDF2	4 of 5	-